QIAGEN Supplementary Protocol

Triplex to 5-plex real-time RT-PCR analysis using the QuantiFast® Pathogen RT-PCR +IC Kit on the Rotor-Gene® Q

This protocol describes how to use the QuantiFast Pathogen RT-PCR +IC Kit to perform real-time RT-PCR analysis in triplex, 4-plex, or 5-plex format using the Rotor-Gene Q real-time cycler.

If you would like to perform duplex RT-PCR (target gene and internal control), please refer to the protocol on page 26 of the *QuantiFast Pathogen RT-PCR +IC Handbook*. Duplex RT-PCR requires slightly different primer and probe concentrations, and the cycling time can be faster.

IMPORTANT: Please consult the "Safety Information" and "Important Notes" sections in the *QuantiFast Pathogen RT-PCR +IC Handbook* before beginning this procedure. QuantiFast Pathogen RT-PCR +IC Kits are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

Equipment and reagents

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For safety information on the additional chemicals mentioned in this protocol, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- QuantiFast Pathogen RT-PCR +IC Kit (cat. nos. 211452 and 211454)
- Primers and hydrolysis (TaqMan®) probes for detection of the pathogen target should be purchased from an established oligonucleotide manufacturer. Primers should be of standard quality, and probes should be HPLC purified. Lyophilized primers and probes should be dissolved in TE buffer to provide a stock solution of 100 µM; concentration should be checked by spectrophotometry (for details, see Appendix A in the *QuantiFast Pathogen RT-PCR +IC Handbook*). Primer and probe stock solutions should be stored in aliquots at –20°C. Probe stock solutions should be protected from exposure to light.
- Nuclease-free (RNase/DNase free) consumables. Special care should be taken to avoid nuclease contamination of all reagents and consumables used to set up PCR for sensitive detection of pathogen nucleic acids.
- Cooling device or ice
- Rotor-Gene Q cycler
- PCR tubes or Rotor-Disc® for the Rotor-Gene cycler

- **Optional**: Trizma® base and EDTA for preparing TE buffer for storing primers and probes (see Appendix A in the QuantiFast Pathogen RT-PCR +IC Handbook). Use RNase/DNase-free water and plastic consumables to prepare TE buffer.
- **Optional**: Internal Control RNA (High conc.; cat. no. 211492). This highly concentrated internal control template is available separately and can be used for optional control of the purification procedure by addition to the sample lysate or lysis buffer.

Important points before starting

- Read "Guidelines for effective multiplex assays" in the *QuantiFast Pathogen RT-PCR +IC Handbook*.
- Always start with the cycling conditions specified in this protocol. The Internal Control Assay has been optimized for multiplexing with pathogen assays generating amplicon lengths of up to150 bp. For PCR products > 150 bp, different cycling conditions may improve results. For details, see Appendix A of the *QuantiFast Pathogen RT-PCR +IC Handbook*.
- Choose reporter dyes and quenchers compatible with the Internal Control Assay (see "Selecting dyes and instrument setup" in the *QuantiFast Pathogen RT-PCR +IC Handbook*).
- We strongly recommend testing the performance of new primer–probe sets in individual assays before combining them in a multiplex assay. Check their concentration and integrity before starting (refer to Appendix A in the *QuantiFast Pathogen RT-PCR +IC Handbook)*.
- Make sure that at least one positive control, as well as one negative control (water, PCR grade), are included per PCR run.
- The PCR **must** start with an initial incubation step of 5 min at 95°C to activate HotStarTaq® *Plus* DNA Polymerase.
- Optimal analysis settings are a prerequisite for accurate quantification data. For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Things to do before starting

- For ease of use, we recommend preparing a 20x primer–probe mix for each of your targets containing target-specific primers and probe. Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions this way, see Appendix E in the *QuantiFast Pathogen RT-PCR +IC Handbook*.

Procedure

1. **Thaw 5x QuantiFast Pathogen Master Mix, primer and probe solutions, RNase-free water, template nucleic acids (isolated viral or bacterial DNA), optional standards, and references. Mix the individual solutions.**

   Standards should be diluted in QuantiTect® Nucleic Acid Dilution Buffer to an appropriate concentration to enable the use of 5–12.5 µl per reaction.

2. **Prepare a reaction mix for the required number of reactions according to Table 1. It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed.**

   Typically, reaction setup can be done at room temperature (15–25°C). However, it is recommended to keep samples, controls, and Internal Control DNA on ice or in a cooling device.

   Note: for RT-PCR, 100x QuantiFast Pathogen RT Mix should be taken from –15 to –30°C immediately before use, always kept on ice, and returned to –15 to –30°C immediately after use.

Table 1. Reaction mix for triplex, 4-plex, or 5-plex analysis

Component	Volume*	Final concentration
QuantiFast Pathogen Master Mix, 5x	5 µl	1x
QuantiFast Pathogen RT Mix, 100x	0.25 µl	1x
20x primer–probe mix 1†	1.25 µl	0.1 µM forward primer 1‡ 0.1 µM reverse primer 1‡ 0.1 µM probe 1§
20x primer–probe mix 2†	1.25 µl	0.1 µM forward primer 2‡ 0.1 µM reverse primer 2‡ 0.1 µM probe 2§
20x primer–probe mix 3†	1.25 µl	0.1 µM forward primer 3‡ 0.1 µM reverse primer 3‡ 0.1 µM probe 3§
For 4-plex assays only: 20x primer–probe mix 4†	1.25 µl	0.1 µM forward primer 4‡ 0.1 µM reverse primer 4‡ 0.1 µM probe 4§
For 5-plex assays only: 20x primer–probe mix 5†	1.25 µl	0.1 µM forward primer 5‡ 0.1 µM reverse primer 5‡ 0.1 µM probe 5§
Internal Control Assay, 10x	2.5 µl	1x
Internal Control RNA, 10x	2.5 µl	1x
RNase-free water	Variable	Variable
Template RNA (added at step 4)	Variable	Variable
Total reaction volume	**25 µl***	–

* If you require a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly.

† For ease of use, we recommend preparing a 20x primer–probe mix for each of your targets containing target-specific primers and probe.

‡ A final primer concentration of 0.1 µM is optimal. Depending on the assay design and pathogen target sequence, performance may be improved by increasing the primer concentration up to 0.3 µM. Before adapting primer concentration, verify the concentration of the primer solutions.

§ A final probe concentration of 0.1 µM gives satisfactory results in most cases.

¶ See "Reconstitution and use of Internal Control Assay and Internal Control RNA" in the *QuantiFast Pathogen RT-PCR +IC Handbook*.

Note: If the Internal Control RNA has been added to the sample lysate or lysis buffer to control purification and amplification, do not add Internal Control RNA to the reaction mix, but add RNase-free water instead.

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into the PCR tubes or wells of a Rotor-Disc.**

   For optimal multiplex performance, we recommend using a total reaction volume of 25 µl. If you require a different reaction volume, adjust the amount of reaction mix including all reaction components accordingly. Make sure to evaluate the performance of pathogen detection in multiplex amplification with the Internal Control in relation to the performance of the pathogen-specific primer–probe set in a singleplex assay.

4. **Add template nucleic acids to the individual PCR wells and mix thoroughly.**

5. **Program the real-time cycler according to Table 2.**

   Data acquisition should be performed during the combined annealing/extension step.

   For the Rotor-Gene Q, we recommend determining the fluorescent range for your pathogen target(s). For the Internal Control Assay, a fixed gain of +9 should be used. For details on adjusting fluorescence sensitivity on Rotor-Gene Q cyclers, see Appendix B of the *QuantiFast Pathogen RT-PCR +IC Handbook*.

Table 2. Cycling conditions for triplex, 4-plex, or 5-plex analysis

Step	Time	Temperature	Additional comments
Reverse transcription into cDNA	**20 min**	**50°C**	RNA is reverse transcribed
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step.
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions.**
Denaturation	**15 s**	**95°C**	
Annealing/extension	**45 s**	**60°C**	Combined annealing/extension step with fluorescence data collection.
Number of cycles	40–50		The number of cycles depends on the amount of template DNA.

6. **Place the Rotor-Disc in the real-time cycler and start the PCR cycling program.**

7. **Perform data analysis.**

   Before performing data analysis, specify the analysis settings. Select the analysis settings (i.e., baseline settings and threshold values) separately for pathogen and Internal Control assay. Note that optimal analysis settings are a prerequisite for accurate detection of data.

QIAGEN Supplementary Protocol

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN handbooks can be requested from QIAGEN Technical Service or your local QIAGEN distributor. Selected handbooks can be downloaded from www.qiagen.com/literature. Material safety data sheets (MSDS) for any QIAGEN product can be downloaded from www.qiagen.com/Support/MSDS.aspx.

www.qiagen.com

Australia ■ 1-800-243-800
Austria ■ 0800/281010
Belgium ■ 0800-79612
Brazil ■ 0800-557779
Canada ■ 800-572-9613
China ■ 0086-21-3865-3865
Denmark ■ 80-885945
Finland ■ 0800-914416
France ■ 01-60-920-930
Germany ■ 02103-29-12000
Hong Kong ■ 800 933 965
Ireland ■ 1800 555 049
Italy ■ 800-787980
Japan ■ 03-6890-7300
Korea (South) ■ 1544 7145
Luxembourg ■ 8002 2076
Mexico ■ 01-800-7742-639
The Netherlands ■ 0800 0229592
Norway ■ 800-18859
Singapore ■ 65-67775366
Spain ■ 91-630-7050
Sweden ■ 020-790282
Switzerland ■ 055-254-22-11
UK ■ 01293-422-911
USA ■ 800-426-8157

Sample & Assay Technologies